REMARKS

After entry of this amendment, claims 1-8, 10-13, and 22-23 are pending.

Claim rejections under 35 U.S.C. § 103(a)

Claims 1-8, 10-13, and 22-23 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Wöber et al. (U.S. Patent Mo. 6,124,110) ("Wöber") in view of a number of additional references cited by the Examiner. *See* Office Action at page 2. The Examiner alleges that this combination of references renders the presently claimed invention obvious. Applicants respectfully traverse.

As set forth in M.P.E.P. § 2143, [t]o establish a *prima facie* case of obviousness, *three* basic criteria must be met. *First*, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *Second*, there must be a reasonable expectation of success. *Finally*, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). In order to establish a *prima facie* case, all three criteria must be met.

The present invention is directed to a kit comprising the following components and attributes as claimed: (1) a lyophilized tissue factor (TF)/phospholipid (PL)-complex; and (2) a lyophilized mixture comprising $CaCl_2$ and a thrombin substrate comprising a fluorescent label. Addition of an aqueous solution to the lyophilized mixture leads to formation of a clear, precipitate-free solution.

Failure to teach or suggest all the claim limitations

Applicants reassert that the presently claimed invention is not obvious in view of the cited art for at least the following reason: the combination of references does not teach or suggest "a lyophilized mixture comprising $CaCl_2$ and a thrombin substrate comprising a fluorescent label, wherein the lyophilized mixture forms a clear solution when dissolved in an aqueous solution". As previously explained, there is no hint or suggestion in any of the

references that the substrates are lyophilized as a mixture with $CaCl_2$. Rather, the substrates and $CaCl_2$ are distinct components of the reactions which are added individually to a reaction mixture from their respective stock solutions. (*See, e.g.*, Wöber *et al.* at col. 4, line 47 to col. 5, line 40; Varadi *et al.* at page 2375, second full paragraph; and Lawson *et al.* at page 4836, col. 2, third full paragraph). Because a lyophilized mixture of a fluorescent substrate and $CaCl_2$ is not disclosed, the references necessarily also fail to teach a lyophilized mixture that forms a clear solution when dissolved in aqueous solution. Accordingly, the combination of references does not teach or suggest every element of the present claims. For this reason alone, a *prima facie* case of obviousness has not been made by the Examiner. Furthermore, even if all the elements of the claimed invention were disclosed, a skilled artisan would have no motivation to combine the references and have no reasonable expectation of success in combining the references as explained below.

Lack of motivation to combine

The fluorescent substrates of the claimed invention are not soluble in aqueous solutions. As set explicitly set forth in Lawson *et al.*, the fluorescent substrate must first be dissolved in an organic solvent such as DMSO prior to use (*see, e.g.*, page 4836, col. 2, second full paragraph). The Applicants provide as additional evidence, the enclosed Analytical Data Sheet for Z-Gly-Gly-Arg-AMC · HCl and scientific publications which show that the substrates of the present invention are insoluble in aqueous solution and must be first dissolved in methanol or a solution containing DMSO. Consistent with this insolubility in aqueous solutions, the instant specification explains that the addition of $CaCl_2$ to a fluorescent substrate in solution leads to formation of a precipitate (*see, e.g.*, page 9, paragraph 28 to page 10, paragraph 29).

Given the insolubility of the substrates in aqueous solution and the precipitation of the substrate from aqueous solutions in the presence of $CaCl_2$, a skilled artisan would have no motivation to lyophilize a suspension of the substrate with $CaCl_2$ with the intention of later reconstituting the dried material because she would have not have an expectation that a clear solution usable in an assay would result from the reconstitution. Rather, she would expect that a suspension would likely result from the addition of an aqueous buffer to the lyophilized material.

Furthermore, because the substrate preparations described in some of the references already exist as dry powders (without $CaCl_2$), there would be even less motivation for the skilled artisan to lyophilize a solution of the substrate in the presence of $CaCl_2$. There would be no reason for the skilled artisan to reconstitute the dried substrate in an aqueous buffer, add $CaCl_2$ to the solution (and experiencing precipitation), only to re-lyophilize the mixture, with the expectation that a precipitate would form again upon reconstitution in aqueous solution. Lyophilization is an involved and time consuming form of drying and requires specialized equipment. Instead of going through such an involved procedure, the skilled artisan would be motivated to add $CaCl_2$ to the solution of the substrate and proceed immediately to perform an assay without lyophilization. A skilled artisan would not recognize any obvious advantage to lyophilizing the substrate with $CaCl_2$, as claimed in the present invention, and would not be motivated to perform this procedure. The references are silent on any benefit that would result from performing this additional step. Thus, for the reasons discussed above, the references provide no motivation or suggestion for combination to arrive at the presently claimed invention.

No reasonable expectation of success

Moreover, the skilled artisan would have no reasonable expectation of success by combining the references as suggested by the Examiner. As discussed above, the addition of $CaCl_2$ to a fluorescent substrate in aqueous solution leads to formation of a precipitate. Accordingly, the skilled artisan would expect that lyophilization of a fluorescent substrate in the presence of $CaCl_2$ would result in a precipitate when such a lyophilized powder is reconstituted with an aqueous buffer or water, not the clear solution as presently claimed.

In view of the foregoing remarks, Applicants submit that a *prima facie* case of obviousness has not been established and the presently claimed invention is nonobvious and thus patentable over the cited references. Accordingly, Applicants request withdrawal of the rejection under 35 U.S.C. §103.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at 925-472-5000.

Respectfully submitted,

Gene H. Yee

Gene H. Yee
Reg. No. 57,471

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
GHY/lls
60643449 v1

Analytical Data Sheet

Lot No. 0538073

Product No.	**4002155.0250** **(I-1140.0250)**		
Product	**Z-Gly-Gly-Arg-AMC · HCl**		
Formula (net)	$C_{28}H_{33}N_7O_7$	*M.W. (net)*	579.6 g/mol

Tests	**Results**
Appearance	White powder
Solubility	50 mg/ml in methanol (clear, colorless solution)
CHN-Analysis	Calc. as 1 HCl x 0.5 H_2O Theory C = 53.80 % Found C = 53.52 % H = 5.66 % H = 5.81 % N = 15.69 % N = 15.42 %
Identity (TLC)	n-Butanol/acetic acid/H_2O 4/2/2 (v/v/v) Chloroform/methanol/acetic acid 32 % 5/3/1 (v/v/v) Plate: silicagel 60 F_{254} Detected by: UV, ninhydrin, chlorine-tolidine
Purity (TLC)	> 99 %
Assay	92.7 %
Water content	1.4 %
Content	Free 7-Amido-4-methylcoumarin < 0.1 %
Comments	Originates from bulk batch 121616

Date: May 11, 1999

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Biochemistry

Detection and partial characterization of a chymostatin-sensitive endopeptidase in transformed fibroblasts

(protease inhibitor/membrane/plasminogen activator)

JILL O'DONNELL-TORMEY* AND JAMES P. QUIGLEY†

Department of Microbiology and Immunology, State University of New York Downstate Medical Center, Brooklyn, New York 11203

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ABSTRACT **A chymostatin-sensitive step in the release of plasminogen activator from transformed fibroblasts has been described recently. By using synthetic peptidyl substrates, we have detected and characterized a chymostatin-sensitive peptidase activity in chicken embryo fibroblasts transformed by Rous sarcoma virus. The activity represents a neutral endopeptidase that exhibits phenylalanine specificity and is inhibited by diisopropyl fluorophosphate. A detailed inhibitor profile of the enzyme activity shows that it is distinct from other chymotrypsin-like phenylalanine-preferring peptidases. The endopeptidase activity in transformed fibroblasts is increased over that of parallel cultures of normal fibroblasts. The mechanism of enzyme inhibition by chymostatin is indicated by these studies, and the possible role of the enzyme in modulating plasminogen activator secretion is discussed.**

The mechanism whereby specific polypeptides are processed and secreted from cells is well established (1–3). The secretory events in this pathway have been defined for a number of specific proteins. However, the mode of release of some proteins into the extracellular milieu is not entirely consistent with the features of this pathway. The properties of one such protein, the serine protease plasminogen activator (PA) that is released in enhanced amounts from a variety of activated and transformed cells (4), indicate that it may not be released via the classical secretory pathway. The cellular form of PA, in contrast to the soluble released form of the enzyme, has been shown to be firmly associated with smooth plasma membrane-like elements of the cell (5). The cell-associated form of PA does not appear to be located within membrane-enclosed vesicles because treatments that readily release vesicle-enclosed enzymes fail to release or solubilize PA (5). In addition, treatment of Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts (RSVCEF) with colchicine at concentrations that inhibit the secretion of other proteins and the movement of secretion granules (6–9) does not inhibit the release of PA (10). Finally, inhibition of protein synthesis for short periods of time, which has no effect on the secretion of established proteins (11), rapidly inhibits the release of PA from RSVCEF (10). Thus, PA appears to exist as a firmly bound membrane-associated enzyme that is actively released into the extracellular medium as a soluble enzyme by an as yet undefined mechanism. It has been suggested that PA, similar to some tumor-specific proteins, is "shed" from the surface of the malignant cell (12), but conclusive evidence for a PA-shedding phenomenon is still lacking. We have determined that in cultures of RSVCEF the appearance of PA in the extracellular medium is inhibited by the protease inhibitor chymostatin (13). This inhibition of PA release is accompanied by concomitant accumulation of the cell-associated form of PA. The effect is specific, is dose and time dependent, and appears to involve the aldehyde moiety of the chymostatin molecule (13). By using specific fluorescent substrates, we have found a chymostatin-sensitive enzyme associated with a membrane fraction isolated from RSVCEF.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Culture. Cultures of chicken embryo fibroblasts were prepared, maintained, and infected with RSV as described (5).

Fluorescence Spectroscopy. The release of 7-amino-4-methylcoumarin (AMC) from the fluorescent substrates Phe-AMC, succinyl (Suc)-Ala-Ala-Phe-AMC, Suc-Ala-Ala-Pro-Ala-AMC, and benzyloxycarbonyl (Cbz)-Gly-Gly-Arg-AMC was measured in a spectrofluorimeter at an excitation wave length of 380 nm and an emission wave length of 460 nm (14). The standard system used to measure hydrolysis of the various substrates consisted of 0.5 ml of a 0.4 mM solution of substrate in 5% dimethyl sulfoxide (Me_2SO) 0.05 M Hepes, pH 7.4, containing 10–100 μg of membrane protein. The linear release of AMC was recorded over 2–15 min and the amount (μmol) of AMC released was calculated against a standard solution of AMC.

Membrane Isolation. The preparation and fractionation of cellular homogenates was as described (5). A membrane fraction was isolated by high-speed centrifugation of a postnuclear supernatant; the resulting pellet is referred to as the total membrane fraction. Protein content (15), PA activity (5), and endopeptidase activity were determined for all fractions. The membrane fraction contained a majority of the cellular PA and endopeptidase activity and 25–30% of the cellular protein.

Materials. Suc-Ala-Ala-Phe-AMC and other peptidyl substrates were purchased from Bachem Fine Chemicals (Torrance, CA). Chymostatin, leupeptin, antipain, and elastatinal were gifts from Walter Troll (New York University School of Medicine). Benzamidine, ε-aminocaproic acid, nitrophenylguanidinobenzoate, tosylphenylalanine chloromethyl ketone (Tos-PheCH$_2$Cl), dithiothreitol, diisopropyl fluorophosphate (iPr_2P-F), 1,10-phenanthroline, EDTA, EGTA, and iodoacetamine were purchased from Sigma. α-1-Antichymotrypsin was obtained from James Travis (University of Georgia, Athens). *N*-Benzoyl-l-phenylalaninal (Bz-Phe-al) was a gift of Richard Schultz (Loyola University Stritch School of Medicine, Maywood, IL). Cbz-Gly-Leu-Phe-Ch$_2$Cl was provided by James Powers (Georgia Institute of Technology, Atlanta).

Abbreviations: RSV, Rous sarcoma virus; RSVCEF, Rous sarcoma virus-transformed chicken embryo fibroblasts; PA, plasminogen activator; iPr_2P-F, diisopropyl fluorophosphate; AMC, 7-amino-4-methylcoumarin; Suc, succinyl; Cbz, benzyloxycarbonyl; Me_2SO, dimethyl sulfoxide; Tos-PheCH$_2$Cl, tosylphenylalanine chloromethyl ketone; Bz-Phe-al, *N*-benzoyl-l-phenylalaninal.

* Present address: Dept. of Cellular Immunology, Rockefeller University, New York, NY 10021.
† To whom reprint requests should be addressed.

RESULTS

Detection of a Chymostatin-Sensitive Activity in RSVCEF. The release of PA from cultures of RSVCEF is inhibited by treatment of the cultures with the protease inhibitor chymostatin (13). A chymostatin-sensitive enzymatic activity has been detected in the total membrane fraction isolated from RSVCEF by using the fluorogenic peptide substrate Suc-Ala-Ala-Phe-AMC. Proteases or peptidases that have phenylalanine specificity will cleave on the COOH-terminal side of phenylalanine to release the fluorescent group AMC. The presence of the NH_2-terminal succinyl blocking group precludes the activity of amino peptidase-initiated sequential hydrolysis of the substrate. As shown in Fig. 1 *A* and *B*, a membrane preparation isolated from RSVCEF exhibits an activity capable of hydrolyzing this substrate that is both time and concentration dependent. Chymostatin inhibits the activity in a dose-dependent manner (Fig. 1*C*), yielding essentially total inhibition at a concentration of 100 μg/ml (0.17 mM). In separate experiments, it was shown that optimal enzyme activity occurred at pH 7.5–8.1, with little or no activity below pH 6 or above pH 9 (data not shown).

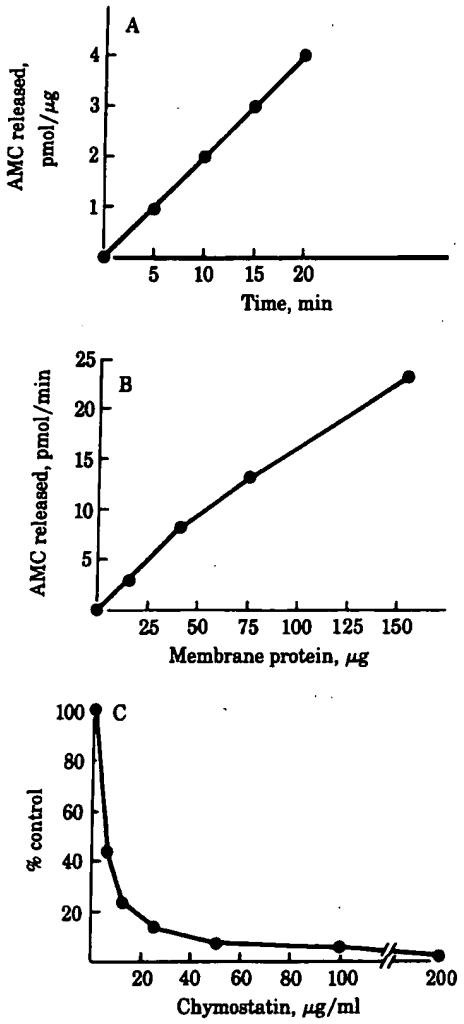

FIG. 1. Detection of a chymostatin-sensitive peptidase activity in a RSVCEF membrane fraction. A 0.5-ml assay mixture containing 0.4 mM Suc-Ala-Ala-Phe-AMC in 5% Me_2SO/0.05 M Hepes, pH 7.4, was used to detect peptidase activity. (*A*) Thirty-eight micrograms of total membrane fraction was added to the assay mixture and the release of AMC was measured at the indicated times. (*B*) Various amounts of membrane protein were added to the assay mixture, release of AMC was monitored for 10 min, and the amount of AMC released per minute was calculated. (*C*) Forty-six micrograms of total membrane fraction was added to the assay mixture in the presence of various concentrations of chymostatin, the rate of AMC release was measured, and the percent inhibition by chymostatin was calculated.

Cleavage Site in Suc-Ala-Ala-Phe-AMC. Release of AMC from Suc-Ala-Ala-Phe-AMC could be due to several enzymatic activities. Direct cleavage on the COOH-terminal side of phenylalanine by a specific endopeptidase would release AMC. However, an endopeptidase exhibiting alanine specificity could also cleave the substrate on the COOH-terminal side of either of the alanine residues, generating Ala-Phe-AMC, Phe-AMC, or both as products. These products, no longer having a blocked amino terminus, could be hydrolyzed by aminopeptidases, resulting in the release of free AMC. As shown in Table 1, the membrane preparation contains an aminopeptidase activity that hydrolyzes an unblocked Phe-AMC substrate. This activity, however, is inhibited by 1,10-phenanthroline and is relatively insensitive to chymostatin. In contrast, the activity measured with Suc-Ala-Ala-Phe-AMC is insensitive to 1,10-phenanthroline and highly sensitive to chymostatin (Table 1), indicating that exopeptidase activity is not involved in the membrane-catalyzed release of AMC. Further evidence that the release of AMC from Suc-Ala-Ala-Phe-AMC is not part of a multistep reaction is that the membrane preparation contains no detectable activity capable of cleaving at alanine residues when the substrate Suc-Ala-Ala-Pro-Ala-AMC is used (data not shown).

The total membrane preparation exhibits other peptidase activities. The fluorescent substrate Cbz-Gly-Gly-Arg-AMC also is hydrolyzed by the membrane preparation (Table 1). This activity, however, is insensitive to chymostatin but is strongly inhibited by the protease inhibitor antipain and thus distinct from the AMC-releasing activity detected when Suc-Ala-Ala-Phe-AMC is used. The chymostatin-sensitive activity in the membrane preparation, therefore, appears to be a specific endopeptidase that has phenylalanine specificity.

K_m and K_i Values for the Endopeptidase Activity. The effect of substrate concentration on the endopeptidase activity in the absence and the presence of various concentrations of chymostatin is shown in Fig. 2. A double reciprocal plot of the data (*B*) indicates a K_m of 0.5 mM for Suc-Ala-Ala-Phe-AMC. A Dixon (16) plot (*C*) indicates a K_i for chymostatin of 5.4 μg/ml (9.2×10^{-6} M). The various plots in Fig. 2 indicate that chymostatin is behaving as a competitive inhibitor, in that high substrate concentration affords protection from chymostatin inhibition. Additional experiments, however, suggest that the inhibition of the endopeptidase by chymostatin is more complex than that of a simple competitive inhibitor. If the membrane fraction is incubated with chymostatin at 20 μg/ml for 2 min, the endopeptidase activity is inhibited 70–80%. When this chymostatin/membrane mixture is diluted 1:20 with buffer and the diluted mixture is incubated for 15 min to allow for any reversal of chymostatin binding, the endopeptidase activity is inhibited only 28%, indicating significant reversibility. However, if the endopeptidase is incubated with chymostatin at 20 μg/ml for 30 min or more and then diluted 1:20 to 1:50 with buffer, little or no reversibility occurs and the enzyme remains 70% inhibited. These data are inconsistent with those for a classical competitive reversible inhibitor and suggest that binding of chymostatin to the enzyme during the short times (as used to generate the data in Fig. 2) can be inhibited by high concentrations of substrate but that, during prolonged incubation, the aldehyde moiety (phenylalaninal) in chymostatin reacts with the enzyme and irreversibly inhibits it. We have shown previously

Table 1. Membrane peptidase activities measured with various fluorescent substrates

	Final conc., μg/ml	AMC released, (pmol/μg of protein)/hr					
		Suc-Ala-Ala-Phe-AMC		Phe-AMC		Cbz-Gly-Gly-Arg-AMC	
		Specific activity	% control	Specific activity	% control	Specific activity	% control
Control		4.81	100	9.48	100	2.87	100
Chymostatin	50	1.24	26	8.52	90	3.41	119
	200	0.39	8	7.20	76	2.56	89
1,10-Phenanthroline	50	5.53	115	6.35	67	NT	—
	200	4.43	92	1.42	15	NT	—
Antipain	50	4.81	100	NT	—	0.77	27

Inhibitor solutions were prepared at various concentrations, and equal volumes were added to the assay mixture to establish the indicated inhibitor concentration (conc.). At the same concentration of membrane protein and the sensitivity used to measure AMC release from these substrates, no activity capable of hydrolyzing Suc-Ala-Ala-Pro-Ala-AMC was detected (data not shown). NT, not tested.

(13) that reduction of the aldehyde in chymostatin eliminates its antiproteolytic activity.

Inhibitor Profile of RSVCEF Endopeptidase. The effects of several protease inhibitors on the endopeptidase-catalyzed hydrolysis of Suc-Ala-Ala-Phe-AMC are summarized in Table 2. Effectors of thiol and metallo proteases, inhibitors of trypsin-like enzymes, and the microbial inhibitors elastatinal and bestatin had no effect on the endopeptidase activity. Each of these compounds also was incubated with the RSVCEF membranes for 30 min prior to the addition of Suc-Ala-Ala-Phe-AMC and still exhibited no inhibitory effect (data not shown). The only treatments that resulted in significant inhibition of AMC release were chymostatin and iPr_2P-F addition. Incubation of the membranes with 1 mM iPr_2P-F for 90 min inhibits the rate of hydrolysis to 47% of the control rate. Incubation of the membranes for 60 min with 2 mM iPr_2P-F inhibits the activity 75%. Thus, the membrane endopeptidase is inhibited by iPr_2P-F but is not highly sensitive to this specific inhibitor of serine proteases, since, under these conditions, chymotrypsin is inhibited 90–100%.

Membrane Endopeptidase Distinct from Other Phenylalanine-Hydrolyzing Proteins. Chymotrypsin and the cellular proteases cathepsin G and chymase are also capable of cleaving the COOH-terminal side of phenylalanine in synthetic substrates and several groups have reported the effects of synthetic and naturally occurring inhibitors on the hydrolytic capacity of these enzymes (17–25). To determine whether the RSVCEF membrane endopeptidase was an enzymatic activity similar or different from cathepsin G, chymase, and chymotrypsin, the effects of several highly specific inhibitors on the membrane-catalyzed hydrolysis of Suc-Ala-Ala-Phe-AMC were determined. As shown in Table 3, the RSVCEF endopeptidase has an inhibitor pattern distinct from that of the other three enzymes. The membrane endopeptidase is most strongly inhibited by chymostatin but considerable inhibition of the activity also is obtained with the tripeptide chloromethylketone, Cbz-Gly-Leu-Phe-CH_2Cl. Bz-Phe-al, Tos-PheCH_2Cl, and α-1-antichymotrypsin have little or no effect on the membrane endopeptidase. In contrast, chymotrypsin is markedly inhibited by all five compounds; chymase is sensitive to α-1-antichy-

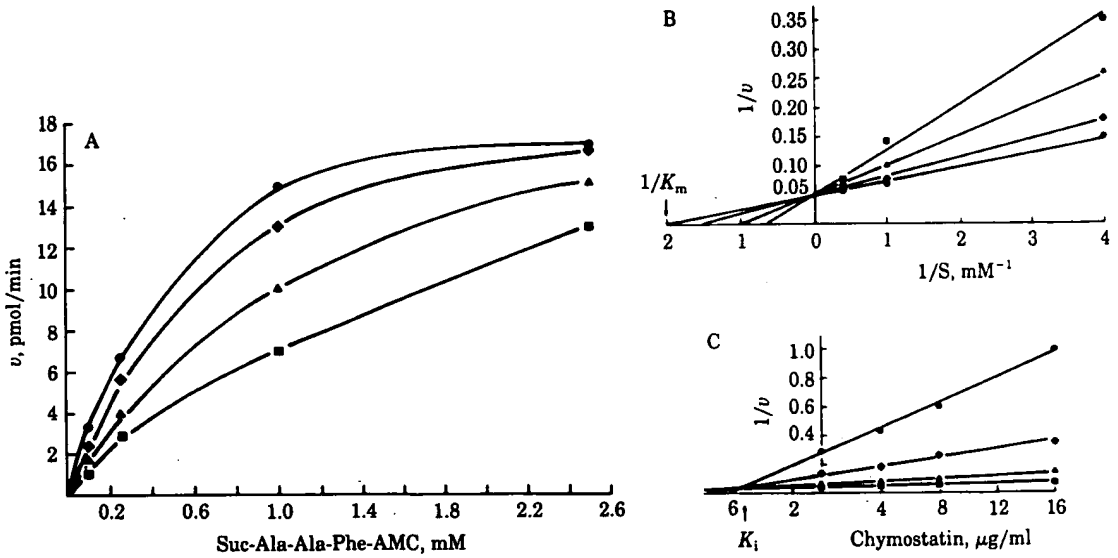

FIG. 2. Endopeptidase activity at different substrate concentrations in the absence and presence of chymostatin. (*A*) Chymostatin at 0 (●), 4 (◆), 8 (▲), and 16 (■) μg/ml was incubated with various concentrations of Suc-Ala-Ala-Phe-AMC (S) in 0.5 ml of a mixture containing 5% Me_2SO/0.05 M Hepes, pH 7.4. Twenty-five micrograms of membrane protein was added, the release of AMC was monitored for 5 min, and the rate of hydrolysis (v) was calculated as pmol of AMC released per min. (*B*) A double reciprocal plot of the data yielding a K_m value of 0.5 mM. (*C*) Dixon (16) plot of the data (Suc-Ala-Ala-Phe-AMC: ●, 0.10; ◆, 0.25; ▲, 1.0; ■, 2.5 mM); the intercept of the lines yields a K_i value for chymostatin of 5.4 μg/ml.

Table 2. Effects of various protease inhibitors on the endopeptidase-catalyzed hydrolysis of Suc-Ala-Ala-Phe-AMC

Addition	Concentration	% control
None		100
Dithiothreitol	5 mM	108
Iodoacetamide	10 mM	98
EDTA	5 mM	113
EGTA	5 mM	109
Benzamidine	5 mM	95
ε-Aminocaproic acid	5 mM	114
Nitrophenylguanidino benzoate	40 μM	100
Leupeptin	200 μg/ml	94
Elastatinal	200 μg/ml	108
Bestatin	200 μg/ml	94
Chymostatin	50 μg/ml	14
	200 μg/ml	3
iPr_2P-F		
30 min	1 mM	69
90 min		47
60 min	2 mM	25

Solutions of inhibitors were prepared such that addition of equal volumes of inhibitor solutions to the assay mixture established the indicated concentration. Inhibitors were added directly to the substrate (0.4 mM) along with 50–200 μg of membrane protein and the rate of AMC release was monitored. To measure the effect of iPr_2P-F, 150 μg of membrane protein was first incubated at room temperature with 1 or 2 mM iPr_2P-F. At the indicated times, an aliquot (50 μg of protein) was added to the substrate and the rate of AMC release was monitored.

motrypsin and Tos-PheCH_2Cl; and cathepsin G, although inhibited by chymostatin and Cbz-Gly-Leu-Phe-CH_2Cl, is also sensitive to Tos-PheCH_2Cl and α-1-antichymotrypsin.

Endopeptidase Activity in Normal Chicken Embryo Fibroblasts and RSVCEF. The endopeptidase activities in cell lysates prepared from parallel cultures of normal chicken embryo fibroblasts and RSVCEF are shown in Table 4. The level of enzyme in normal chicken embryo fibroblasts is increased approximately 3-fold following transformation by RSV. The activity in both cultures is inhibited 90% by chymostatin at 50 μg/ml.

DISCUSSION

The inhibition by chymostatin of PA release from cultures of RSVCEF (13) has led to the identification and partial characterization of a chymostatin-sensitive enzyme activity in RSVCEF. This activity appears to be due to a membrane endopeptidase that cleaves on the COOH-terminal side of phenylalanine in synthetic peptide substrates (Table 1). The inhibitor profile indicates that the endopeptidase is not a thiol or metallo enzyme but rather a serine peptidase inhibitable by iPr_2P-F (Table 2). The enzyme activity, when compared with those of purified serine proteases, does not exhibit a high sensitivity to iPr_2P-F. However, the heterogeneous nature of the membrane fraction that contains the endopeptidase may influence its reactivity with iPr_2P-F. The membrane-associated form of PA isolated from these cells likewise requires relatively high levels of iPr_2P-F for inhibition compared with the levels required for the purified soluble form of PA (26).

Table 4. Comparison of endopeptidase activities in normal and transformed chicken embryo fibroblasts

	Endopeptidase-specific activity
Normal	6.5 ± 2.6
RSVCEF	20.9 ± 4.1

Parallel cultures of normal and transformed fibroblasts were harvested during exponential growth. Cellular homogenates were prepared, and multiple aliquots of the homogenates were assayed for endopeptidase activity and protein content. Results are expressed as pmol of AMC released/μg of protein per hr and represent mean ± SD

The RSVCEF endopeptidase appears to be distinct from chymotrypsin and two other phenylalanine-preferring chymotrypsin-like cellular enzymes, cathepsin G and chymase (Table 3). Although some similarities do exist between the inhibitor profile of the endopeptidase and the profile of these other proteolytic enzymes, distinct discrepancies are apparent. These data suggest that the RSVCEF endopeptidase represents an as yet undescribed enzyme activity; however, it is important to point out that a crude form of the endopeptidase was assayed in these studies and compared with highly purified forms of the other enzymes (Table 3). A truly valid comparison must await purification of the endopeptidase.

A chymostatin-sensitive membrane-associated endopeptidase also has been reported by Zimmerman and co-workers (27, 28). This activity was detected by deoxycholate-solubilize pancreatic microsomal membranes by using the same synthetic substrate, Suc-Ala-Ala-Phe-AMC. The microsomal endopeptidase, however, was reported to cleave the substrate between alanine and phenylalanine and to be inhibited by 1,10-phen-

Table 3. Effects of specific inhibitors on the RSVCEF membrane endopeptidase and other phenylalanine-preferring proteases

Inhibitor	Conc.	% inhibition			
		Endopeptidase	Chymotrypsin	Cathepsin G	Chymase
α-1-Antichymotrypsin	7.6 μg/ml	0	100	Inhibition (17)	Inhibition (17)
Chymostatin	0.1 mM	94	100	Inhibition (18)	—
(R_1-Gly-Leu-Phe-al)	1.0 mM	100	100		
Bz-Phe-al	0.1 mM	0	30	—	—
	1.0 mM	6	93		
Cbz-Gly-Leu-Phe-CH_2Cl	0.1 mM	66	73	Inhibition (19)	—
	1.0 mM	93	96		
Tos-PheCH_2Cl	0.1 mM	0	33	No inhibition (20)	Inhibition (21, 22)
	1.0 mM	3	94	Inhibition (23, 24)	

Inhibitors at the indicated concentrations (conc.) were incubated with membrane (10 μg) for 30 min at 22°C in 0.45 ml of 0.05 M Hepes/5% Me_2SO, pH 7.4. A control containing membranes but no inhibitor was incubated in parallel. Fifty microliters of 0.01 M Suc-Ala-Ala-Phe-AMC was added to initiate the reaction and the release of AMC was monitored. The effects of the inhibitors on chymotrypsin-mediated hydrolysis of Suc-Ala-Ala-Phe-AMC were measured in parallel experiments. Effect of the inhibitors on chymase and cathepsin G were taken from the indicated references. R_1, [(*S*)-(1-carboxy-2-phenylethyl)carbonyl]-α-[2-iminohexahydro-4(*S*)-pyrimidyl].

anthroline (28). In contrast, the RSVCEF endopeptidase cleaves on the COOH-terminal side of phenylalanine and is insensitive to 1,10-phenanthroline (Table 2) and thus is distinct from the microsomal endopeptidase.

The mechanism of inhibition by chymostatin of the RSVCEF endopeptidase has been suggested by these studies. The data in Table 3 indicate that the effect of chymostatin on the enzyme is not just due to the terminal phenylalaninal residues since the simple phenylalaninal compound, Bz-Phe-al elicits no inhibitory effect. Similarly, the terminal phenylalanine chloromethyl ketone in Cbz-Gly-Leu-Phe-CH_2Cl is not solely responsible for enzyme inhibition because the endopeptidase is insensitive to the simple phenylalanine chloromethyl ketone compound, Tos-PheCH_2Cl. The inhibitory effects of both chymostatin and Cbz-Gly-Leu-Phe-CH_2Cl therefore suggest that the tripeptide structure Gly-Leu-Phe is necessary to position the inhibitor at the active site of the enzyme. Once bound to the active site, either the aldehyde or chloromethyl ketone group can react to inactivate the enzyme. The kinetic data in Fig. 3 are consistent with a competition for active site binding between the Gly-Leu-Phe-al moiety of chymostatin and the Ala-Ala-Phe-AMC of the substrate. The time-dependent occurrence of irreversible inhibition is likewise consistent with aldehyde-mediated inhibition of the enzyme following chymostatin binding.

It is tempting to speculate that the RSVCEF endopeptidase activity described herein is involved in the mechanism of PA release. The release of PA from a firmly bound membrane state to a soluble extracellular form does not appear to proceed via the classical secretion pathway. The release of PA from these cells, however, is sensitive to chymostatin and the inhibitory effect of chymostatin on PA release appears to be due specifically to the antiproteolytic activity of chymostatin (13). The only detectable chymostatin-sensitive peptidase activity in RSVCEF cultures is the described endopeptidase (Table 1). The endopeptidase activity is enhanced after transformation of chicken embryo fibroblasts by RSV (Table 4), an event that also leads to increased production of PA (4). A number of other cell cultures have been examined including B16 melanoma and human fibrosarcoma HT-1080. Similar to the RSVCEF cultures, the release of PA from these mammalian cultures is inhibited by chymostatin and the cells also possess a chymostatin-sensitive endopeptidase activity (unpublished data). These observations provide further support for a role of the endopeptidase in PA release. However, there are some unresolved questions that prevent the establishment of a direct link between endopeptidase activity and PA release. First, the concentration of chymostatin that is required to completely inhibit the endopeptidase activity (50–100 μg/ml) is 15–30% of that needed in culture medium to inhibit PA release (300 μg/ml). This may be due to an inability to estimate the effective concentration of chymostatin at the cell surface because chymostatin is relatively insoluble in aqueous culture medium (13). Also, an inaccessibility of the cellular endopeptidase to exogenous chymostatin may require high concentrations of the compound in the extracellular medium. A second unresolved question is that the natural substrate for the endopeptidase has not been established. Although the cellular form of PA is firmly bound to RSVCEF membranes (5), the molecular mass of the released extracellular form of PA is identical (within 1,000 daltons) to that of the cell-associated form (10). Therefore, if the endopeptidase functions proteolytically to release PA from its membrane association, it would not appear to cleave a large membrane-bound segment of the PA molecule. Instead, the endopeptidase may cleave other membrane proteins that directly or indirectly interact with PA. The cleavage of these proteins could perturb the association of PA with the membrane, resulting in the release of soluble PA into the extracellular medium. However, until such a putative substrate is found, the link between endopeptidase activity and PA release remains circumstantial. Nevertheless, the circumstantial evidence that links the two enzymes—i.e., chymostatin sensitivity—has allowed for the detection of this apparently unique endopeptidase activity in transformed fibroblasts. Its purification, now feasible because of its specific inhibitor profile and reactivity toward defined synthetic substrates, should allow for both the identification of its natural substrate(s) and the elucidation of its role in the subtle, yet influential, protein perturbations that occur in normal and transformed cell membranes.

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A New Fluorogenic Substrate for Plasmin

By Patricia A. PIERZCHALA, Conrad P. DORN and Morris ZIMMERMAN
Merck, Sharp and Dohme Research Laboratory, P.O. Box 2000, *Rahway, NJ* 07065, *U.S.A.*

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A new fluorogenic peptide substrate for plasmin, 7-(*N*-succinoylalanylphenylalanyllysylamido)-4-methylcoumarin trifluoroacetate salt, was prepared that can be used in a simple and direct assay. The results obtained by the assay method are linear over a wide range of enzyme concentrations and sensitive enough to detect as little as 10^{-5} CTA units of plasmin. By making use of the inhibitor Trasylol and the differences in kinetic constants, plasmin can be specifically assayed even in the presence of the plasminogen activator thrombin, as well as in culture fluids from HeLa cells.

Plasmin is a trypsin-like enzyme whose role appears to be the dissolution of blood clots. It is present in the blood as the zymogen, plasminogen, which is incorporated between the filaments when the fibrin matrix is formed. Plasminogen is converted into plasmin by the cleavage of a single Arg–Val bond through the action of a proteolytic enzyme such as plasminogen activator (Stroud, 1974; Robbins & Summaria, 1976). Plasmin breaks down the fibrin matrix by splitting Arg–X or Lys–X bonds.

There presently exist several different methods for assaying plasmin, including the use of protein substrates, e.g. fibrin or casein (Astrup & Mullertz, 1952; Johnson *et al.*, 1969), peptide esters (Sherry *et al.*, 1966; Bell *et al.*, 1974), peptide amides, e.g. nitroanilides (Mattler & Bang, 1977), and peptide 4-methoxy-2-naphthylamides (Clavin *et al.*, 1977). The standard fibrin plate method, although being highly sensitive, requires long incubation times and, as with the methods involving the use of other protein substrates, does not allow kinetic studies to be carried out conveniently. Peptide esters are generally not as specific for individual proteinases as are amides (Feinstein *et al.*, 1973; Mattler & Bang, 1977). Measurement of the rate of hydrolysis of 4-methoxy-2-naphthylamides is either indirect via coupling to a dye or direct by a fluorescent assay with lessened sensitivity. There exists a need for a direct plasmin assay method that is rapid and extremely sensitive to allow detailed kinetic studies.

Workers in this laboratory have previously reported (Zimmerman *et al.*, 1978) that the synthetic fluorogenic peptide Cbz-Gly-Gly-Arg(F_3Ac)-AMC is a highly sensitive substrate for plasminogen activator. Utilizing the same fluorescent leaving group, 7-amino-4-methylcoumarin, we have prepared a plasmin substrate, Suc-Ala-Phe-Lys(F_3Ac)-AMC, that can be used to assay extremely low concentrations of plasmin. We report here the use of this substrate in detecting plasmin impurities in samples of urokinase and thrombin as well as the detection of small amounts of plasmin in culture fluids from HeLa cells.

Abbreviations used: Suc, succinoyl; F_3Ac, trifluoroacetyl; Cbz, benzyloxycarbonyl; Boc, t-butoxycarbonyl; AMC, 7-amino-4-methylcoumarin; CTA units, Committee on Thrombolytic Agents of the NIH units, based on the digestion of a standard batch of α-casein (Johnson *et al.*, 1969); ID_{50}, 50%-inhibitory dose, concentration that inhibits 50% of the enzyme's activity under assay conditions described in the text; Tes, 2-{[-2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethane sulphonic acid.

Materials and Methods

Materials

Tes was obtained from Pierce, Rockford, IL, U.S.A. All the amino acids used were obtained from Chemalog, South Plainfield, NJ, U.S.A. The 7-amino-4-methylcoumarin was prepared as described by Zimmerman *et al.* (1976), with the following modification to remove trace fluorescent contaminants. 7-Amino-4-methylcoumarin was converted into the crystalline tosylate salt by suspending it in hot ethanol, adding toluene-*p*-sulphonic acid and allowing crystals to form by slow cooling. The salt was then converted into the free amine by suspending it in 0.5M-sodium acetate, washing with water and drying either *in vacuo* or by azeotropic distillation with toluene. The 7-(*N*-Cbz-glycylglycylargininamido)-4-methylcoumarin trifluoroacetate was prepared as described by Zimmerman *et al.* (1978). The 7-Boc-alanylphenylalanyl-ε-Cbz-lysylamido-4-methylcoumarin was prepared by a similar procedure.

To the mixed anhydride prepared at −30°C from

18mmol of Boc-ε-Cbz-lysine, 20mmol of *N*-methylmorpholine and 20mmol of isobutyl chloroformate in 100ml of dimethylformamide was added 20mmol of 7-amino-4-methylcoumarin. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue dissolved in 100ml of methylene dichloride. After two extractions with 50ml of 2.5M-HCl, the organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Chromatography of the residue on 600g of silica gel and elution with ether/hexane (1:1, v/v) followed by ether gave 2.3g (23.8% yield) of 7-(Boc-ε-Cbz-lysylamido)-4-methylcoumarin (m.p. 139–142°C, decomp.); elemental analysis: found: C, 64.58; H, 6.59; N, 7.80; calc. for $C_{29}H_{35}N_3O_7$ (mol.wt. 537.59): C, 64.79; H, 6.56; N, 7.82%.

Selective removal of the *N*-Boc protecting group was accomplished by dissolving 4.2mmol of the above material in 20ml of trifluoroacetic acid at 0°C, allowing the reaction mixture to warm to room temperature, followed by removal of the solvent *in vacuo* to give the trifluoroacetate salt of 7-(ε-Cbz-lysylamido)-4-methylcoumarin. This crude salt and 5mmol of *N*-methylmorpholine were added to the mixed anhydride prepared from 4mmol of Boc-Phe, 4.4mmol of *N*-methylmorpholine and 4.4mmol of isobutyl chloroformate in 50ml of dimethylformamide at −30°C. The reaction mixture was stirred cold for 5min, then allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue was extracted between 200ml of ethyl acetate and 100ml of 0.25M-HCl. The organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Chromatography of the residue on 500g of silica gel and elution with ethyl acetate/methylene dichloride (1:1, v/v) gave 2.77g (96.3%) of 7-(Boc-phenylalanyl-ε-Cbz-lysylamido)-4-methylcoumarin (m.p. 175–177°C, decomp.); elemental analysis: found: C, 66.28; H, 6.51; N, 7.94; calc. for $C_{38}H_{44}N_4O_8$ (mol.wt. 684.76); C, 66.65; H, 6.48; N, 8.18%.

Deblocking of 3.3mmol of the above dipeptide amide in 20ml of trifluoroacetic acid under the conditions described above gave the trifluoroacetate salt of 7-(phenylalanyl-ε-Cbz-lysylamido)-4-methylcoumarin, which was used in the following step without purification.

To the mixed anhydride prepared from 3.5mmol of Boc-Ala-OH, 4.0mmol of *N*-methylmorpholine and 4.0mmol of isobutyl chloroformate in 50ml of dimethylformamide at −30°C was added the above salt in 25ml of dimethylformamide containing 4.0mmol of *N*-methylmorpholine. The reaction mixture was stirred cold for 10min, then allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue was taken up between 100ml of ethyl acetate and 50ml of 1M-HCl. The aqueous layer was separated and extracted with 50ml of ethyl acetate. The combined ethyl acetate layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting solid was slurried with ether/hexane (1:1, v/v) and filtered to give 2.36g (94.7%) of 7-(Boc-alanylphenylalanyl-ε-Cbz-lysylamido)-4-methylcoumarin (m.p. 168–170°C, decomp.); elemental analysis: found: C, 65.11; H, 6.65; N, 9.36; calc. for $C_{41}H_{49}N_5O_9$ (mol.wt. 755.84): C, 65.15; H, 6.53; N, 9.27%.

The t-butoxycarbonyl protecting group was removed from the tripeptide amide as described above to yield the trifluoroacetate salt of Ala-Phe-ε-Cbz-Lys-AMC. To prepare the succinoyl derivative, 0.26mmol of the above material was dissolved in dimethylformamide, and then 0.26mmol of *N*-methylmorpholine and 0.26mmol of succinic anhydride were added. The reaction was stirred overnight. The solvent was then removed *in vacuo* and the residue was washed with water to yield Suc-Ala-Phe-ε-Cbz-Lys-AMC. The ε-benzyloxycarbonyl protecting group on the lysine was removed by refluxing in hot trifluoroacetic acid for 20min followed by removal of the solvent *in vacuo*.

MeOSuc-Ala-Phe-ε-Cbz-Lys-AMC was prepared by adding to 0.13mmol of the above trifluoroacetate salt, dissolved in dimethylformamide, 0.33mmol of *N*-methylmorpholine and 0.20mmol of 3-carbomethoxypropionyl chloride. The reaction mixture was stirred overnight. The solvent was removed *in vacuo* and the residue washed with water. The ε-benzyloxycarbonyl protecting group was removed as described above, giving MeOSuc-Ala-Phe-Lys-(F_3Ac)-AMC. The purity of these substances was determined by t.l.c. and amino acid analysis.

Highly purified urokinase, thrombin and human plasmin were obtained from Dr. Thomas Finlay, Department of Biochemistry, New York University Medical Center, New York, NY, U.S.A. A separate sample of purified urokinase was obtained from Dr. Alan Johnson, Department of Medicine, New York University Medical School, New York, NY, U.S.A. Trasylol was obtained from FBA Pharmaceuticals, New York, NY, U.S.A.

Culture fluids from HeLa cells were prepared as described by Zimmerman *et al.* (1978).

Fluorimetric assays

Enzyme assays were conducted at 24°C with the substrate in 0.05mM-Tes/NaOH buffer, pH7.5, and 20% (v/v) dimethyl sulphoxide in a final volume of 0.5ml. Dimethyl sulphoxide at concentrations up to 20% had no inhibitory or stimulatory effects on plasmin. To determine the ID_{50} of Trasylol on thrombin, 0.32mM-Ala-Phe-Lys-AMC was used with 22.2g of thrombin, and 0.12mM-Ala-Phe-Lys-AMC was used with 5×10^{-4} CTA units of plasmin.

Fluorescence of the 7-amino-4-methylcoumarin

generated was determined as described by Zimmerman *et al.* (1976). A Perkin–Elmer model 204A spectrofluorometer was standardized daily so that 10nM-7-amino-4-methylcoumarin gave 1.0 chart division (0–100 scale).

Determination of K_m

The kinetic constants were calculated from a double-reciprocal plot by the Lineweaver–Burk method, and are based on rate determinations at six different substrate concentrations. Correlation coefficients were greater than 0.99. The substrate concentrations used to determine the various K_m values generally ranged from 0.5 to 4 times the K_m value obtained.

Results

The trifluoroacetyl derivatives of Ala-Phe-Lys-AMC, Suc-Ala-Phe-Lys-AMC and MeOSuc-Ala-Phe-Lys-AMC are all excellent plasmin substrates that can be used in a simple, direct, fluorimetric assay method. The limit of detection with the succinoyl derivative is 10^{-5} CTA units of plasmin in a reaction time of 5min, and the rate of hydrolysis is linear over at least a 100-fold range of enzyme concentrations. The succinoyl and methoxysuccinoyl substrates exhibit similar kinetic constants. However, the unblocked substrate has a K_m of 0.045mM, which is 10-fold lower, and a specific activity approximately half of that for either of the blocked peptides (Table 1).

Table 1. *Kinetic constants of the hydrolysis of 7-amino-4-methylcoumarin substrates by plasmin, urokinase and thrombin*
Assays were performed as described in the text with 5×10^{-4} and 5×10^{-3} CTA units of plasmin, 0.2μg and 0.01μg of urokinase and 22.2μg and 0.222μg of thrombin with the lysine and arginine substrates respectively. The specific activities are expressed as μmol of 7-amino-4-methylcoumarin released/min per mg of protein for urokinase and thrombin and as μmol of 7-amino-4-methylcoumarin released/min per CTA unit of plasmin. The specific activities were calculated from the V values and enzyme concentrations. –, Not determined.

	Plasmin		Urokinase		Thrombin	
	K_m (mM)	Sp. activity	K_m (mM)	Sp. activity	K_m (mM)	Sp. activity
Ala-Phe-Lys-AMC	0.045	210	0.90	100	0.80	9
Suc-Ala-Phe-Lys-AMC	0.40	370	0.80	62	–	–
MeOSuc-Ala-Phe-Lys-AMC	0.44	470	–	–	–	–
Cbz-Gly-Gly-Arg-AMC	0.45	10	0.13	1600	0.11	475

Table 2. *Detection of plasmin in the presence of urokinase, thrombin or plasminogen activator*
Assays were carried out as described in the text with 0.045mM-Ala-Phe-Lys-AMC, 0.32mM-Cbz-Gly-Gly-Arg-AMC and 0.8mM-Suc-Ala-Phe-Lys-AMC as substrates and 1.4μM-Trasylol where indicated. The urokinase and thrombin concentrations per assay were 50ng and 222ng respectively. The asterisk (*) indicates that the assay mixture contained plasmin: urokinase*, 1.25×10^{-5} CTA units of plasmin was added to the assay, and thrombin*, 6.0×10^{-5} CTA units of plasmin was added. The activities are expressed as nmol of 7-amino-4-methylcoumarin released/min per μg of protein for urokinase and thrombin and nmol of 7-amino-4-methylcoumarin released/min for urokinase*, thrombin* and HeLa cells. –, Not determined.

	Ala-Phe-Lys-AMC		Cbz-Gly-Gly-Arg-AMC		Suc-Ala-Phe-Lys-AMC	
	–Trasylol	+Trasylol	–Trasylol	+Trasylol	–Trasylol	+Trasylol
Urokinase	–	–	1013	960	–	–
Urokinase*	0.06†	0.0	–	–	–	–
Thrombin	0.0	–	387	401	–	–
Thrombin*	0.44	0.0	–	–	–	–
HeLa (0.1ml)	–	–	–	–	0.08	0.02
HeLa (0.2ml)	–	–	0.26	0.30	0.15	0.05
HeLa (0.4ml)	–	–	0.54	0.54	0.21	0.06

† Amount of plasmin added is at the detection limit for this assay.

Highly purified urokinase and thrombin also hydrolyse Ala-Phe-Lys-AMC, but at a much lower rate and with K_m values of 0.9mM and 0.8mM respectively (Table 1). The large difference between the K_m with plasmin and that with either urokinase or thrombin has enabled us to assay specifically for plasmin in the presence of urokinase or thrombin. Samples of urokinase and of thrombin, deliberately contaminated with 1.25×10^{-5} and 6.0×10^{-5} CTA units of plasmin respectively, were assayed with 0.045mM-Ala-Phe-Lys-AMC. In both cases the activity of the small amount of plasmin was able to be measured (Table 2).

With the substrate Ala-Phe-Lys-AMC, the ID_{50} of Trasylol with plasmin was found to be $<0.05\mu M$ and with thrombin $>23\mu M$. Zimmerman *et al.* (1978) have reported that Trasylol will completely inhibit plasmin with no effect on urokinase activity. With the plasminogen activator substrate Cbz-Gly-Gly-Arg-AMC they report an ID_{50} of $>12\mu M$ with urokinase. However, when urokinase was assayed with Ala-Phe-Lys-AMC 50% inhibition was observed on the addition of only $1.4\mu M$-Trasylol. A similar result was obtained with a different sample of purified urokinase. When the assay was repeated with Cbz-Gly-Gly-Arg-AMC as the substrate, no inhibition of urokinase activity was found on Trasylol addition (Table 2), thus suggesting a slight contamination of 'pure' urokinase with plasmin. When a mixture of plasmin and thrombin or plasmin and urokinase was assayed with Ala-Phe-Lys-AMC, the enzyme activity was totally inhibited by the addition of $1.4\mu M$-Trasylol (Table 2). This result supports the observation that the plasmin activity was able to be measured specifically in the presence of other similar enzymes.

When assaying plasmin activity in crude extracts, one of the blocked substrates must be used to avoid non-specific substrate hydrolysis by aminopeptidases. Culture fluids from HeLa cells were assayed with 0.8mM-Suc-Ala-Phe-Lys-AMC and 0.32mM-Cbz-Gly-Gly-Arg-AMC. As shown in Table 2, both substrates were hydrolysed. To ensure that the activity observed with the high concentration of Suc-Ala-Phe-Lys-AMC was due to plasmin rather than plasminogen activator, Trasylol was added to the assay mixture. It inhibited most of the activity (70%) with the lysine substrate, but had no effect on the hydrolysis rate with the arginine substrate (Table 2).

Discussion

We have described a new fluorogenic peptide substrate allowing the detection of extremely low concentrations of plasmin. The assay method is rapid and direct, with no observable spontaneous hydrolysis of the substrate.

The sequence chosen was based on the chloromethyl ketone inhibition data reported by Kettner *et al.* (1977), which showed Ac-Ala-Phe-Arg-CH_2Cl to inhibit plasmin 475-fold better than it inhibits urokinase. Since plasmin prefers lysine at the bond cleaved, Ala-Phe-Lys-AMC was synthesized.

Unlike the 4-methoxy-2-naphthylamide substrate described by Clavin *et al.* (1977), Suc-Ala-Phe-Lys-AMC can be used directly to detect as little as 10^{-5} CTA units of plasmin, compared with the 5×10^{-2} CTA unit detection limit that they report. Nieuwenhuizen *et al.* (1977) have also reported a fluorogenic peptide substrate for plasmin and plasminogen activator with the use of a β-naphthylamide leaving group; however, it is a better substrate for urokinase than for plasmin. Alternately, Ala-Phe-Lys-AMC has a 20-fold lower K_m with plasmin than with either urokinase or thrombin, thus enabling plasmin contamination in either preparation to be readily measured. The sensitivity and specificity of Ala-Phe-Lys-AMC is such that trace amounts of another proteinase, presumably plasmin, were detected in supposedly pure preparations of urokinase. Confirmation of these results, achieved through the use of the macromolecular inhibitor Trasylol, which completely inhibits plasmin activity with little of no effect on urokinase or thrombin, demonstrates the utility of combining the use of this new substrate with Trasylol to check the purity of enzymes isolated from biological fluids.

The succinoyl and methoxysuccinoyl substrates both have much higher K_m values than the unblocked substrate. Possibly the carboxy group and its methyl ester interfere with binding (assuming $K_m = K_s$), and studies on whether extending the peptide chain will optimize the substrate should be undertaken.

The blocked substrate Suc-Ala-Phe-Lys-AMC was used when assaying crude extracts to avoid any possible aminopeptidase action on the substrate. Although there is only a 2-fold difference in K_m, discrimination between plasmin and urokinase was possible in the presence of Trasylol. Culture fluids from malignant cells (HeLa) were found to contain low concentrations of plasmin in addition to plasminogen activator. This method can be applied to other cell systems to assay directly and specifically for plasmin.

Suc-Ala-Phe-Lys-AMC has been shown to be a much more highly sensitive and specific plasmin substrate than any other reported. By using both Cbz-Gly-Gly-Arg-AMC and Suc-Ala-Phe-Lys-AMC it is now possible to assay the same cell fluids for both plasminogen activator and plasmin activity.

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Direct fluorescent assay of urokinase and plasminogen activators of normal and malignant cells: Kinetics and inhibitor profiles

(fluorogenic substrates/protease inhibitors/protease secretion/plasmin/trypsin-like enzymes)

M. ZIMMERMAN*, J. P. QUIGLEY†, B. ASHE*, C. DORN*, R. GOLDFARB†, AND W. TROLL‡

* Merck Institute for Therapeutic Research, Rahway, New Jersey, 07065; † State University of New York, Downstate Medical Center, Brooklyn, New York, 11203; and ‡ Department of Environmental Medicine, New York University Medical Center, New York, New York, 10016

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ABSTRACT **A direct rate assay for plasminogen activator has been developed using a synthetic fluorogenic peptide substrate, 7-(*N*-Cbz-glycylglycylargininamido)-4-methylcoumarin trifluoroacetate. The assay correlates well with the standard ^{125}I-labeled fibrin plate assay using highly purified urokinase, culture fluids from WI-38, Chinese hamster ovary or HeLa cells, or Rous sarcoma virus-transformed chick fibroblasts as the source of plasminogen activator. The assay is sensitive, rapid, and linear throughout a wide range of enzyme concentrations. With this substrate it is possible to determine inhibitor profiles for the various plasminogen activators, independently of the interfering potential of plasmin. All of the enzymes tested are inhibited by leupeptin and antipain but not by the related aldehydes, elastatinal and chymostatin. The macromolecular inhibitors soybean trypsin inhibitor and trasylol have little or no effect on the plasminogen activators tested. This substrate should be useful for the study of the effect of various agents on functional changes in cells secreting this enzyme and also should allow kinetic measurements of potential inhibitors.**

The enhanced production of plasminogen activator (PA) activity has been shown to be a characteristic of many different cell types. The intracellular and extracellular levels of PA have been demonstrated to be substantially elevated in malignant cells in culture (1–6), cells treated with a tumor promoter (7), activated macrophages (8, 9), established cell lines (10, 11), granulosa cells during ovulation (12), embryonic cells during differentiation (13, 14), and hormone-treated uteri (15). The standard system used for measuring PA in these cells is an indirect, two-step assay in which plasminogen is incubated with a source of PA and the plasmin activity generated is quantitated by using fibrin, casein, or protamine as substrates (16–19). There is a need, however, for a simple, sensitive, direct assay that allows both rapid measurement and kinetic analysis of PA, independent of plasmin generation. In addition, the presence of two proteases of similar specificities in the two-step assay precludes the screening of potential PA inhibitors.

A series of synthetic fluorogenic substrates, specific for a number of serine proteases, utilizing the leaving group 7-amino-4-methylcoumarin (AMC) has been described (20, 21). In a continuation of this approach, we have now prepared a synthetic peptide specific for the cleavage site of PA, incorporating the same leaving group. This compound is Cbz-Gly-Gly-Arg-AMC. We report here the use of this substrate in the direct fluorescent assay of PA from normal and malignant cells, some kinetic parameters of these enzymes, and the effect of various low and high molecular weight protease inhibitors on the various enzymes. The various PAs were analyzed by the direct fluorescent technique in parallel with the indirect standard ^{125}I-labeled fibrin plate assay using purified plasminogens from both canine and bovine sources.

MATERIALS AND METHODS

Enzymes. Highly purified urokinase (UK) was obtained from Alan Johnson, Department of Medicine, New York University Medical School. Human plasmin was obtained from M. Mosseson, Department of Medicine, Downstate Medical Center, SUNY. Purified human granulocyte elastase and cathepsin G were prepared as described (22). Hog pancreatic elastase, chromatographically purified, was obtained from Miles Laboratories. Bovine α-chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp.

Inhibitors. Trasylol was obtained from FBA Pharmaceuticals, and soybean trypsin inhibitor (SBTI) was from Worthington Biochemical Corp. Leupeptin, antipain, elastatinal, and chymostatin were provided by the U.S.–Japan Cooperative Cancer Research Program.

Preparation of PA-Containing Culture Fluids. Primary cultures of chicken embryo fibroblasts were prepared, maintained, and infected with Rous sarcoma virus as described (23); these infected cells are designated RSVCEF. Fully transformed cultures of RSVCEF were grown in Eagle's minimal medium supplemented with plasminogen-free fetal bovine serum (24). When the cultures had attained high cell density (1×10^7 cells per 100-mm culture dish), the plates were washed three times with minimal medium and further incubated in serum-free minimal medium. The medium was removed from the cultures every 12 hr and was a source of extracellular PA; it is referred to as harvest fluid (HF). After it was harvested, the HF was immediately centrifuged to remove cells and cellular debris and acidified to pH 3.5 by the addition of 1 M HCl. Ammonium sulfate was added to 70% saturation, and the resulting precipitate was recovered by centrifugation and resuspended in 1/100 the volume of the original HF in 0.05 M glycine·HCl buffer, pH 3.0.

WI-38, HeLa, and Chinese hamster ovary cells were grown in serum-containing medium, washed, and incubated in serum-free Higuchi medium (25). This medium was removed after 24 or 48 hr and was the source of extracellular PA. These HFs were centrifuged, acidified, and concentrated as described for the Rous HF.

All of the HF concentrates were dialyzed for 16 hr against 0.05 M sodium acetate, pH 5.25, to remove the residual ammonium sulfate (at concentrations of 0.1 M and greater, ammonium sulfate inhibits the fluorometric assay). They were

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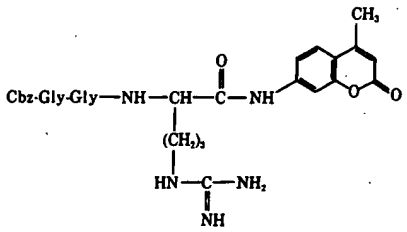
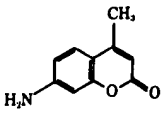
Abbreviations: PA, plasminogen activator; AMC, 7-amino-4-methylcoumarin; UK, urokinase; SBTI, soybean trypsin inhibitor; RSVCEF, Rous sarcoma virus-infected chicken embryo fibroblasts; HF, harvest fluid.

Table 1. Fluorescence properties of substituted 4-methylcoumarins

	Maxima, nm	
	Excitation	Emission
Substrate	325	395
		
Product	345	445
		
Optimum difference for assay purposes	383	455

stored frozen and used as sources of PA in both the fluorescent and fibrin plate assays.

Synthesis of Fluorogenic Substrate. *N*-Cbz-Arg-AMC was prepared essentially as described (21). The compound was purified by chromatography on silica gel (Baker) with $CHCl_3$/MeOH, 70:30 (vol/vol), as the developing solvent. After deblocking with 32% HBr in acetic acid, the Arg-AMC·HBr was coupled to Cbz-Gly-Gly by using isobutylchloroformate (26). Cbz-Gly-Gly was prepared from Cbz-chloride and glycylglycine under Schotten–Baumann conditions. The substrate was purified again on silica gel with $CHCl_3$/MeOH, 90:10 (vol/vol), as the developing solvent. Purity was determined by thin-layer chromatography, amino acid analysis, and nuclear magnetic resonance spectroscopy.

Fluorometric Assay for PA. Enzyme assays with the fluorogenic substrate were conducted at 24° with 0.1–0.5 mM substrate in 0.05 M 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate buffer, pH 7.5/5% (vol/vol) dimethyl sulfoxide; the final volume was 0.5 ml in each assay. Fluorescence of the AMC was monitored continuously by using a Perkin–Elmer 204A spectrofluorometer equipped with a chart recorder. Although both the substrate and its hydrolysis product are highly fluorescent, their excitation and emission wavelengths are distinctly different (20, 21). Activation and emission wavelengths (383 and 455 nm, respectively) were chosen such that AMC retained 20% of its maximal fluorescence but possessed a relative fluorescence 500-fold greater than that of an equimolar amount of Cbz-Gly-Gly-Arg-AMC (Table 1). The instrument was standardized daily so that 10 nM AMC gave 1.0 chart division (0–100 scale).

Fibrin Plate Assay for PA. PA was assayed on ^{125}I-labeled fibrin-coated petri dishes with purified plasminogen-free fibrinogen and purified fetal bovine or dog plasminogen as described (23).

RESULTS

Specificity and Kinetics of the Fluorometric Assay. Cbz-Gly-Gly-Arg-AMC is not only an excellent substrate for UK but also can be used to detect the presence of PA, either directly or after concentration in cultures with lower levels of activity, in the serum-free medium bathing various cells. Using this substrate, we measured PA activity secreted from HeLa, Chinese hamster ovary, and WI-38 cells and RSVCEF.

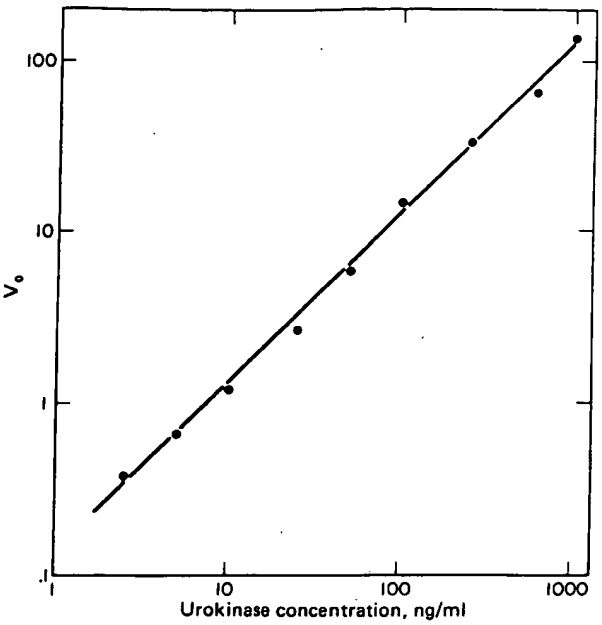


FIG. 1. Initial velocity versus urokinase concentration. Initial velocity (V_0) is expressed as nmol of AMC released per min.

As shown in Fig. 1, the limit of detection of UK with this substrate and a reaction time as short as 5 min is 2.5 ng of protein per ml, and the rate of hydrolysis is proportional to enzyme concentration over at least a 400-fold range. The sensitivity could easily be increased by longer incubation times. The rate of hydrolysis of RSVCEF HF concentrate also is linear over at least a 100-fold range (Fig. 2). The K_m for UK with this fluorogenic substrate is 4×10^{-4} M; with the RSVCEF concentrate it is 6×10^{-4} M.

Neither UK nor the RSVCEF activator hydrolyzed, at a significant rate, the fluorogenic substrates for elastase, trypsin, and chymotrypsin reported previously (11). Conversely, Cbz-Gly-Gly-Arg-AMC is not significantly hydrolyzed by pancreatic elastase, granulocyte elastase, chymotrypsin, or granulocyte cathepsin G. However, like many arginine peptides, the substrate is hydrolyzed by trypsin, thrombin, and plasmin. The possible presence of the latter enzyme is monitored in our assay systems both by the plasminogen dependence

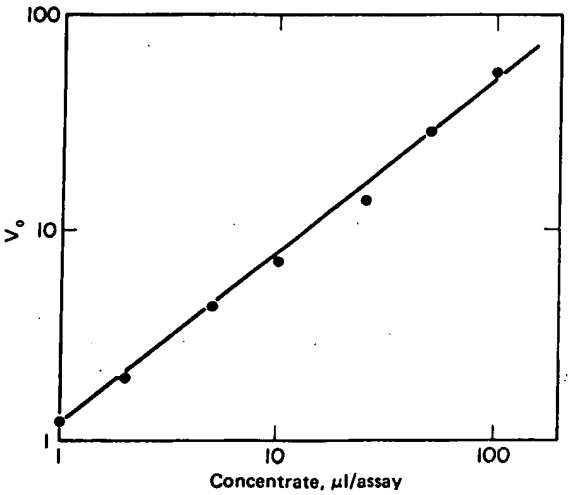


FIG. 2. Initial velocity versus concentration of HF concentrate from RSVCEF. Initial velocity (V_0) is expressed as nmol of AMC released per min.

Table 2. Comparison of fibrinolytic and fluorometric assays for plasminogen activator

Plasminogen	PA	Fibrinolytic assay: Incubation time, hr	^{125}I released, cpm	Ratio RSVCEF/UK
Dog, 7 μg	5 μl RSVCEF	1.5	1,314	1.37
	5 μl UK	1.5	960	
	5 μl RSVCEF	4.0	4,812	1.13
	5 μl UK	4.0	4,263	
	5 μl RSVCEF	7.0	13,550	1.01
	5 μl UK	7.0	13,406	
	10 μl RSVCEF	1.5	2,930	1.15
	10 μl UK	1.5	2,539	
	10 μl RSVCEF	4.0	11,900	1.38
	10 μl UK	4.0	8,607	
	10 μl RSVCEF	7.0	27,887	1.20
	10 μl UK	7.0	23,237	
Fetal bovine, 7 μg	5 μl RSVCEF	1.5	14,863	11.94
	5 μl UK	1.5	1,245	
	10 μl RSVCEF	1.5	20,162	10.16
	10 μl UK	1.5	1,985	

PA	Fluorometric assay: AMC released, nmol/min	Ratio, RSVCEF/UK
10 μl RSVCEF	5.3	1.23
10 μl UK	4.3	
20 μl RSVCEF	14.0	1.31
20 μl UK	10.7	

* UK solution contained 5 μg of protein per ml.

of the fibrin plate assays and the response to inhibitors described below.

Correlation of Fibrinolytic and Fluorometric Assays. We correlated the enzyme activity responsible for the hydrolysis of Cbz-Gly-Gly-Arg-AMC with PA activity measured in the standard fibrinolysis assay. As shown in Table 2, we found an excellent correlation of RSVCEF PA/UK ratios between the fluorometric assay and the fibrin plate assay with dog plasminogen. Fetal bovine plasminogen appears to be much more sensitive to the RSVCEF enzyme. With dog plasminogen, the relative activities of the PAs in concentrates from HeLa, Chinese hamster ovary, and WI-38 cells were determined in the two assays. On the basis of assigning the HeLa concentrate an activity of 1.0, the HeLa/hamster ovary/WI-38 ratio in the fibrinolytic assay was 1.0:1.1:6.9; in the fluorometric assay it was 1.0:0.95:8.5, which is again a good correlation. It should be emphasized that, in the absence of plasminogen, there was little or no detectable fibrinolytic activity with 10 μl of either UK or any of the concentrates added to the fibrinolytic system. Therefore, the activity being detected in these fluids is indeed PA and not plasmin. The fluorogenic substrate, added as a stock concentrate in dimethylsulfoxide, has no effect on the fibrin plate assay; therefore, neither the substrate itself nor the vehicle at the concentrations used is inhibitory to either PA or plasmin.

Inhibitor Studies. Using the fluorogenic substrate, we determined the effect of various specific protease inhibitors on UK and the enzymes derived from various cell lines. Table 3 shows that leupeptin and antipain are inhibitors of both UK and the RSVCEF enzyme with K_i values of approximately the same order of magnitude. These two compounds are competitive inhibitors, based on Dixon plots. On the other hand, neither chymostatin, an inhibitor of chymotrypsin-like enzymes, nor elastatinal, a specific inhibitor of elastase (27), inhibited these activators at a concentration of 100 μg/ml. As expected, both UK and the RSVCEF enzyme were inhibited by diisopropylfluorophosphate.

Table 3. K_i values of leupeptin and antipain with two plasminogen activators

Enzyme	K_i, M: Leupeptin	Antipain
Urokinase	2.6×10^{-5}	6×10^{-5}
RSVCEF PA	1.6×10^{-5}	2×10^{-5}

The response of PAs from various sources to several known inhibitors of trypsin-like enzymes is of considerable interest. As shown in Table 4, all of the enzymes tested are inhibited by both leupeptin and antipain, but in all cases leupeptin is the better inhibitor. UK and the enzymes from HeLa, hamster ovary, and WI38 cells have similar leupeptin/antipain inhibitory ratios but are not inhibited by trasylol or SBTI. The RSVCEF enzyme exhibits a leupeptin/antipain ratio similar to that of the other PAs but is inhibited slightly by SBTI. Plasmin is inhibited by much lower concentrations of all these inhibitors, again clearly demonstrating that we are measuring PA, not plasmin, in the extracellular fluids.

DISCUSSION

We have developed a direct rate assay for PA that uses a synthetic fluorogenic peptide. The assay results correlate with those of the standard fibrin plate assay using culture fluids from various cells as the source of PA. The assay is sensitive, rapid, and allows for the determination of kinetic constants and inhibitor profiles.

The sequence of the synthetic peptide was chosen to optimize the activity with PA. Kettner *et al.* (28) prepared a peptide chloromethyl ketone, with the sequence Ac-Gly-Gly-Arg-CH_2Cl, which inhibits UK 23-fold better than it inhibits plasmin (8), identifying this sequence as being desirable. Huseby *et al.* (29) recently reported the detection of UK activity by using a similar peptide linked to the leaving group 4-methoxy-2-naphthylamine; however, the leaving group was detected indirectly by using a fast blue dye. In another approach to the direct assay of PA, Dano and Reich (30) reported a use of the macromolecular substrate, radiolabeled plasminogen. They determined hydrolysis of this substrate by electrophoresis, measuring the appearance of the heavy and light chains of plasmin.

Use of the substrate reported in this study has allowed us to

Table 4. Inhibition of PAs from various sources and plasmin by inhibitors of trypsin-like enzymes

Enzyme	ID_{50},* μM: Leupeptin	Antipain	Trasylol	SBTI
Urokinase	67	165	>12	>4.5
RSVCEF PA	38	100	>12	1.4
HeLa PA	38	165	>12	>4.5
WI-38 PA	110	165	12	>4.5
CHO PA†	89	200	>12	>4.5
Plasmin	1.1	6.6	0.046	0.0031

Substrate: 0.5 nM Cbz-Gly-Gly-Arg-AMC in 0.05 M pH 7.5 buffer, containing 5% dimethyl sulfoxide.

* 50% inhibitory dose, the concentration that inhibits 50% of the enzyme activity under assay conditions described in text.

† CHO, Chinese hamster ovary.

detect directly PAs from cultures of transformed fibroblasts (RSVCEF), a malignant cell line (HeLa), and two nonmalignant cell types (WI-38 and Chinese hamster ovary). In addition, purified UK was detected and compared to the PAs from the cultures. With all of the PAs tested, the fluorometric assay showed an excellent correlation with the fibrin plate assay (Table 2); however, the fluorometric assays were performed more rapidly and in the absence of plasminogen. The striking difference between the activation of canine and bovine plasminogens by UK and the RSVCEF activator should be noted. However, differences between plasminogens from different sources with respect to their activation by various PAs have been reported (24). The fluorometric assay was demonstrated to be linear throughout a wide range of enzyme concentrations and allowed determination of K_m values with different enzymes for the substrate and K_i values for inhibitors. Thus, with this assay it was possible to establish an inhibitor profile for this important enzyme. The macromolecular inhibitors SBTI and Trasylol had little or no effect on the PAs tested, in agreement with the observations by Dano and Reich (30). On the other hand, we found inhibition with leupeptin and antipain but not with the related aldehydes elastatinal and chymostatin. We also found that leupeptin and antipain were competitive inhibitors.

This direct assay should facilitate monitoring of enzyme purification and studying functional changes in cells as measured by changes in enzyme secretion, as well as the large-scale testing of PA inhibitors independently of the interfering potential of plasmin.

Note Added in Proof. Since this manuscript was initially submitted, a similar substrate for urokinase has been reported (31).

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